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(54) Title: PEPTIDES AND PEPTIDE DERIVATIVES FOR THE TREATMENT OF  $\alpha$ -SYNUCLEIN-RELATED DISEASES

(57) Abstract: The invention provides peptides which comprise a sequence of three to twelve contiguous amino acid residues (the "contiguous sequence") from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein. The contiguous sequence is linked at its N-terminal and/or C-terminal end to one or more further amino acid residue(s) which are more hydrophilic than the amino acid residue to which that end of the sequence is linked in naturally occurring  $\alpha$ -synuclein. The invention also provides derivatives and analogues of such peptides. Peptides, derivatives or analogues of the invention are of use in prevention of  $\alpha$ -synuclein oligomerisation and/or aggregation associated with the diseases known as the synucleinopathies. As a result peptides according to the invention are susceptible of use in the preparation of medicaments, and also in methods of treatment, for prevention and/or treatment of synucleinopathies.

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**PEPTIDES AND PEPTIDE DERIVATIVES FOR THE TREATMENT OF  $\alpha$ -  
SYNUCLEIN-RELATED DISEASES.**

The present invention relates to peptides and their derivatives which are useful for the prevention and/or treatment of the diseases known as "the synucleinopathies" by inhibiting and/or disrupting the aggregation of  $\alpha$ -synuclein and/or NAC.

The synucleins are a family of small proteins (~14 kDa) that are expressed at high levels in nervous tissue. The three members of the family ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -synuclein) are the products of three genes present on different chromosomes (1). The full amino acid sequence of human wild-type  $\alpha$ -synuclein is provided as Sequence ID No. 1 in Figure 1.

Diseases associated with abnormalities in one or more of the synucleins are referred to herein as the 'synucleinopathies' and include some important neurodegenerative conditions, for example Parkinson's disease (PD), dementia with Lewy bodies (DLB), Alzheimer's disease (AD) and multiple system atrophy (MSA). The synucleins are also expressed at abnormally high levels in various tumours (e.g. breast, ovarian) in human cancer (2).

Many of these neurodegenerative diseases are characterised by accumulation of extracellular or intracellular inclusions comprised of insoluble amyloid material.

The first indication of an involvement of  $\alpha$ -synuclein in the pathogenesis of neurodegenerative diseases came from the isolation of one of its proteolytic fragments from purified amyloid of Alzheimer's diseased (AD) brains (2). This  $\alpha$ -synuclein fragment, representing about 10% of the sodium dodecyl sulphate (SDS) insoluble material, was named non-A $\beta$ -component of AD amyloid (NAC). Amino acid sequencing revealed that NAC comprised at least 35 amino acids (provided as Sequence ID No.2 in Figure 1), although the N- terminal residues could not be assigned with certainty because of the specificity of the enzyme used in sequencing.

These 35 amino acids were later shown to correspond to residues 61-95 of a 140 amino-acid precursor (NACP). NACP was found to be identical with the protein called  $\alpha$ -synuclein (3). A clear link with PD was established when it was shown that two different mutations in the  $\alpha$ -synuclein gene were found in rare inherited forms of this disease. One mutation,  $\alpha$ -synuclein(A53T), has been found in certain Italian and Greek families, and results in an Ala<sup>53</sup> to Thr substitution (4). The other mutation,  $\alpha$ -synuclein(A30P), has been found in a family of German origin, and results in an Ala<sup>30</sup> to Pro change (5).

Lesions in the brain known as 'Lewy bodies' and 'Lewy neurites' constitute the main pathological features in the brains of patients with PD and DLB. These Lewy bodies and Lewy neurites contain the  $\alpha$ -synuclein in an unusual fibrillar form (6). Additional immunohistochemical and immunoelectron microscopy studies have shown that  $\alpha$ -synuclein is also associated with pathological lesions in other neurodegenerative diseases, sometimes involving non-neuronal cells, such as the glial cytoplasmic inclusions found in MSA (7). These and other neurodegenerative diseases involving  $\alpha$ -synuclein are collectively known as the 'synucleinopathies'. It has recently been reported that lesions similar to those found in the human synucleinopathies can be created in transgenic animals. The transgenic animals express high levels of human wild-type or mutant  $\alpha$ -synuclein protein and progressively develop many of the pathological conditions associated with synucleinopathies (8,9). These findings appear to implicate  $\alpha$ -synuclein protein deposition in the pathophysiology of the synucleinopathies.

It is believed that the conversion of  $\alpha$ -synuclein into insoluble fibrils is initiated by a conformational or proteolytic modification. It has been suggested that the  $\alpha$ -synuclein aggregation is a nucleation-dependent phenomenon in which the initial insoluble "seed" allows the selective deposition of the inclusions (10).

Recombinant proteins containing the full length 1-140 and shorter peptides containing the hydrophobic region (61-78) of  $\alpha$ -synuclein can self-aggregate *in vitro* to form

amyloid-like fibrils in the absence of other proteins (11). This suggests that the potential to form amyloid resides mainly in the structure of  $\alpha$ -synuclein. Interestingly, the two  $\alpha$ -synuclein mutations appear to accelerate the aggregation process (11, 12). Aggregates formed from wild-type  $\alpha$ -synuclein or from either mutant were thioflavine-S positive, indicative of the presence of aggregates with the  $\beta$ -pleated sheet conformation characteristic of amyloid fibrils (11). Anti-parallel  $\beta$ -sheet structure in wild-type and mutant aggregates has been confirmed by Fourier transform infrared spectroscopy (12). The relationship between the primary structure of  $\alpha$ -synuclein and its ability to form amyloid-like fibrils was analysed by altering the sequence of the protein. Substitution of hydrophobic residues for charged ones in the internal  $\alpha$ -synuclein hydrophobic regions (amino acids 71-82) impaired fibril formation (13), suggesting that  $\alpha$ -synuclein assembly is partially driven by hydrophobic interactions. Indeed, shorter peptides containing the hydrophobic region of  $\alpha$ -synuclein (e.g.  $\alpha$ -synuclein (61-78); (61-95); (1-87) and (1-120)) are more amyloidogenic than the full length (14,15). Secondly, the  $\beta$ -sheet conformation adopted by  $\alpha$ -synuclein protein is crucial in amyloid formation (11,13,16). The importance of hydrophobicity and  $\beta$ -sheet secondary structure on amyloid formation also is suggested by comparison of the sequence of other amyloidogenic proteins (17).

Protein conformation-dependent neurotoxicity is an emerging theme in neurodegenerative disorders such as AD, Huntington's Disease and prion disease (reviewed in 18). A similar conformation-dependent mechanism may also be relevant to synucleinopathies (18, 19). Indeed, it has been shown that aggregates from  $\alpha$ -synuclein and its fragments containing the hydrophobic region 61-78 are toxic to dopaminergic human neuroblastoma SH-SY5Y cells (14).

Currently, there is no cure or treatment that significantly retards the progression of the synucleinopathies. There is therefore an urgent need to develop agents suitable for use in treatment and prevention of these diseases.

It is an object of the present invention to provide agents capable of inhibiting and/or

disrupting the aggregation of  $\alpha$ -synuclein or NAC.

According to the present invention there is provided a peptide comprising a sequence of three to twelve contiguous amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein, linked at the N-terminal and/or C-terminal end of the sequence to one or more further amino acid residue(s) which are more hydrophilic than the amino acid residue(s) to which that end of the sequence is linked in naturally occurring  $\alpha$ -synuclein.

The present invention is based upon the surprising finding that peptides derived from the sequence of amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein can be used to inhibit and/or disrupt aggregation of  $\alpha$ -synuclein or its derivatives such as NAC (apart from where the context requires otherwise all references to the inhibition and/or disruption of aggregation of  $\alpha$ -synuclein should be taken also to apply to the inhibition and/or disruption of aggregation of derivatives of  $\alpha$ -synuclein). This finding is particularly surprising in the light of the prior art cited above which teaches that peptides comprising portions of the amino acid sequence of this region cause synuclein aggregation, and are even more amyloidogenic than native  $\alpha$ -synuclein or NAC.

The contiguous sequence of peptides according to the invention may preferably comprise at least four hydrophobic amino acid residues. The four hydrophobic amino acid residues may preferably be contiguous with one another. The four hydrophobic amino acid residues may, for example, comprise the residues AVVT (residues 69 to 72 of full length naturally occurring synuclein).

As a result of their ability to disrupt and/or inhibit aggregation of  $\alpha$ -synuclein peptides according to the invention are suitable for use in the treatment and/or prevention of synucleinopathies. The peptides are useful for disrupting and/or inhibiting aggregation of "wild-type"  $\alpha$ -synuclein (native form), or mutated, nitrated, phosphorylated or glycosylated forms, or any other naturally occurring modified

forms.

Peptides of the invention comprise a sequence of three to twelve amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein, linked at the N-terminal and/or C-terminal end of the sequence to one or more further amino acid residues which are more hydrophilic than the amino acid residue to which that end of the sequence is linked in naturally occurring  $\alpha$ -synuclein. For purposes of clarity and brevity the sequence of three to twelve amino acid residues from residues 61 to 100 of  $\alpha$ -synuclein is herein referred to as the "contiguous sequence". Furthermore, the further amino acids linked at the end of the "contiguous sequence" are herein referred to as the "replacement" amino acids, and the amino acid residues to which the end of the contiguous sequence are attached in naturally occurring  $\alpha$ -synuclein are referred to as the "substituted" amino acids.

Hydrophilicity/hydrophobicity of amino acids in the context of the invention may be determined in accordance with known hydropathy indexes. An example of a suitable hydropathy index is the Kyte Doolittle hydropathy index, for which values for naturally occurring amino acids are shown in Table 1. In accordance with the index, a hydrophilic amino acid has a negative hydropathy index value and a hydrophobic amino acid has a positive value. In accordance with the invention, an amino acid that is more hydrophilic than another amino acid has the lower hydropathy index value. Thus, by way of illustration, an amino acid having an index value of, say, +1 is more hydrophilic than one having an index value of, say, +2.

Table 1.

Amino Acid	Kyte Doolittle Hydrophathy Index
Alanine	1.8
Arginine	-4.5
Aspartic acid	-3.5
Asparagine	-3.5
Cysteine	2.5
Glutamic acid	-0.4
Glutamine	-3.5
Glycine	-3.5
Histidine	-3.2
Iso-Leucine	4.5
Leucine	3.8
Lysine	-3.9
Methionine	1.9
Phenylalanine	2.8
Proline	-1.6
Serine	-0.8
Threonine	-0.7
Tryptophan	-0.9
Tyrosine	-1.3
Valine	4.2

Preferably the “replacement” amino acid has a hydrophathy index value at least one, preferably at least two, more preferably at least three, and most preferably at least four less than that of the “substituted” amino acid. Most preferably the “replacement” amino acid is a hydrophilic amino acid, as defined by its hydrophathy index.

The contiguous sequence comprises from three to twelve amino acid residues. Preferably the contiguous sequence comprises a maximum of seven amino acid residues, more preferably a maximum of six amino acid residues, even more

preferably a maximum of five amino acid residues, and most preferably a maximum of four amino acid residues.

Preferably the contiguous sequence includes at least two contiguous hydrophobic amino acid residues from amino acid residues 61 to 100 of  $\alpha$ -synuclein. More preferably a peptide according to the invention comprises at least three contiguous hydrophobic amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein. In a preferred embodiment a peptide of the invention comprises the amino acid residues alanine-valine-valine found at residues 69 to 71 of naturally occurring  $\alpha$ -synuclein. In another preferred embodiment a peptide of the invention comprises the amino acid residues alanine-valine-alanine found at residues 76 to 78 of naturally occurring  $\alpha$ -synuclein. In a third preferred embodiment a peptide of the invention comprises the amino acid residues isoleucine-alanine-alanine-alanine found at residues 88 to 91 of naturally occurring  $\alpha$ -synuclein.

Peptides according to the invention may comprise the contiguous sequence linked to one (or more) replacement amino acid(s) at one end of the sequence, the or each replacement amino acid being more hydrophilic than the corresponding amino acid in the naturally occurring  $\alpha$ -synuclein sequence. Alternatively the contiguous sequence may be linked to one (or more) replacement amino acid(s) at each end of the sequence.

Peptides of the invention may comprise a single contiguous sequence linked to one (or more) replacement amino acid(s), or they may comprise multiple contiguous sequences linked to one another by one (or more) replacement amino acid(s). For example a peptide according to the invention may comprise a contiguous sequence linked to a replacement amino acid, the replacement amino acid being in turn linked to a further contiguous sequence.

Examples of peptides in accordance with the invention are shown in the left hand column of Table 2 below, which also identifies the peptide's contiguous sequence, the



residues of  $\alpha$ -synuclein from which the contiguous sequence is derived, and the substitutions made.

**Table 2.**

Peptide, or peptide derivative, of the invention.	Contiguous sequence.	Position of contiguous sequence in $\alpha$ -synuclein.	Substitutions made.
Number 1. RGGAVVTGR	GGAVVTG	67-73	V66→R V74→R
Number 2. RGAVVTGR	GAVVTG	68-73	G67→R V74→R
Number 3. GGAVVTGR	GGAVVTG	67-73	V74→R
Number 4. RGAVVGR	GAVV	68-71	G67→R T72→G G73→R
Number 5. nGnGAVVTnGVTAVA	(i)AVVT (ii)VTAVA	69-72 74-78	G68→nG G69→nG G73→nG
Number 6. nGGAVVTGVTAVA	GAVVTGTAVA	68-78	G68→nG
Number 7. nGnGnAnVnVnTnGR			
Number 8. RGGAVVTGRRRRRR	GGAVVTG	67-73	G67→R V74→R

(In the above table n preceding a residue indicates that the residue is N-methylated, for example nG represents N-methylated glycine).

Peptides according to the invention are effective for inhibiting the aggregation of

soluble synucleins. The peptides are also effective for disrupting existing synuclein aggregates. Peptides of the invention may also be used to disrupt existing synuclein aggregates whilst inhibiting the formation of new aggregates. Preferably peptides of the invention are capable of disrupting and/or inhibiting  $\alpha$ -synuclein protein aggregation even when the  $\alpha$ -synuclein protein concentration is in molar excess compared to the concentration of the peptide.

Derivatives or analogues of the peptides of the invention are also effective for inhibiting and/or disrupting aggregation of synucleins. Therefore, according to a second aspect of the invention there is provided a derivative or analogue of a peptide according to the first aspect of the invention.

Derivatives or analogues of peptides according to the invention may include N-methylated derivatives of the peptides. Such N-methylated derivatives include derivatives in which some or all of the contiguous sequence are N-methylated amino acid residues, and derivatives in which some or all of the replacement amino acids are N-methylated residues.

Derivatives or analogues of peptides according to the first aspect of the invention may further include D-amino acid derivatives of the peptides, peptoid analogues of the peptides, or peptide-peptoid hybrids.

Peptides may be subject to degradation by a number of means (such as protease activity in biological systems). Such degradation may limit their bioavailability, and hence their ability to disrupt and/or inhibit synuclein aggregation. There are a wide range of well established techniques by which peptide derivatives that have enhanced stability in biological contexts can be designed and produced. Such peptide derivatives may have improved bioavailability as a result of increased resistance to protease-mediated degradation.

Preferably a peptide derivative or analogue according to the second aspect of the invention is more protease-resistant than the peptide from which it is derived.

Protease-resistance of a peptide derivative and the peptide from which it is derived may be evaluated by means of well-known protein degradation assays. The relative values of protease resistance for the peptide derivative and peptide may then be compared.

In peptoid residues, the position of the side chain is shifted from the  $\alpha$ -carbon atom to the nitrogen atom [20]. The identity of the side chain is conserved, while the direction in three-dimensional space is changed. These compounds may be considered a combination of backbone and side chain modifications. Peptoid compounds have two properties that make them suitable for use as peptide derivatives/analogues according to the invention:

- (i) In peptoid residues no hydrogen bond involving the NH would be possible.
- (ii) The peptoids are resistance to enzymatic degradation.

Peptoid derivatives of the peptides of the invention may be readily designed from knowledge of the structure of the chosen peptide. Commercially available software may be used to develop peptoid derivatives according to well-established protocols.

It has been reported that a retropeptoid, (in which all amino acids were replaced by peptoid residues in reversed order) is able to mimic a high-affinity binder [21]. A retropeptoid is expected to bind in the opposite direction in the ligand-binding groove, as compared to a peptide or peptoid-peptide hybrid containing one peptoid residue. As a result, the side chains of the peptoid residues are able point in the same direction as the side chains in the original peptide.

This approach using N-methylated amino acids has been used successfully to design peptide-peptoid hybrid peptidomimetic inhibitors for  $\beta$ -amyloid protein and amylin (IAPP) aggregation and toxicity in *vitro* [22, 23].

Peptide-peptoid hybrid peptidomimetics can also be used to disrupt and/or inhibit  $\alpha$ -

synuclein aggregation. Such hybrids comprise peptides in which one or more amino acids have been replaced by the corresponding peptoid residues. Examples of such hybrids include the fifth and sixth peptides listed in Table 2.

In another embodiment of the second aspect of the invention the contiguous sequence comprises D-amino acids. In this case the order of the contiguous sequence is reversed as compared to the section of the sequence of  $\alpha$ -synuclein on which it is based. Thus for example a D-amino acid based peptide derivative with a contiguous sequence based on residues 69 to 71 of  $\alpha$ -synuclein (GAVV) would have a contiguous sequence of VVAG.

Peptides, peptide derivatives and peptide analogues according to the invention may be adapted to facilitate their entry into cells, or across biological barriers (such as the blood brain barrier). Since synucleinopathies commonly involve pathological activity of synucleins in the brain, facilitating the entry of peptides or peptide derivatives of the invention into this tissue is highly desirable.

Methods have been developed for the delivery of exogenous proteins into living cells and across the blood-brain barrier with the help of membrane-permeable carrier peptides such as HIV-1 Tat-(48-60), flock house virus (FHV) coat-(35-49), *Drosophila* Antennapedia-(43-58) and Basic peptides such as octa and hexa arginine peptides. By genetically or chemically hybridising these carrier peptides, the efficient intracellular delivery of various oligopeptides and proteins has been achieved. The efficacy of such approaches is illustrated by the example of the Tat- $\beta$ -galactosidase fusion protein [24], which has a molecular mass as high as 120 kDa. Expression of this fusion protein in mice results in delivery of the biologically active fusion protein to all tissues, including the brain. More recently, it has been reported that polyglutamine binding peptides targeted for intracellular delivery by fusion to TAT retain the ability to inhibit polyglutamine aggregation and cell death in transfected cells [25]. Thus peptides or peptide derivatives of the invention may be adapted in order to increase their bioavailability in cells or tissues by the incorporation of such carrier peptides.

This approach, using carrier peptides to improve availability of peptides, peptide derivatives and peptide analogues of the invention is particularly suitable for allowing the incorporation into tissues or cells of molecules containing unnatural amino acids (e.g. D-amino acids or N-methylated amino acids) or non-peptide derivatives.

Peptides, peptide derivatives and peptide analogues according to the invention may be further modified according to well-known techniques to increase their solubility, or to further aid their ability to inhibit  $\alpha$ -synuclein aggregation. For example, the solubility of a peptide, peptide derivative or peptide analogue according to the invention may be increased by the inclusion of a chain of multiple glycine, lysine or arginine residues at the N-terminal and/or C-terminal end(s) of the molecule (26). The inventors have found that particularly beneficial results may be achieved by the addition of poly-arginine chains, such as hexa-arginine.

The increased solubility achieved by the methods described above may be beneficial in increasing the ability of peptides, peptide derivatives and peptide analogues according to the invention to cross the blood-brain barrier, and may facilitate the uptake of the peptides, derivatives and analogues into biological cells.

Peptides, peptide derivatives and peptide analogues according to the invention can also be used diagnostically in assays to detect and quantify  $\alpha$ -synuclein protein in an *in vitro* sample, such as a sample of biological fluid.

According to a third aspect of the invention there is provided a method of treatment and/or prevention of a synucleinopathy comprising administering to a person in need of such treatment and/or prevention a therapeutically effective amount of a peptide, peptide derivative or peptide analogue according to the first or second aspects of the invention.

Peptides, peptide derivatives and peptide analogues according to the invention are effective in causing the inhibition or disruption of synuclein aggregation associated

with synucleinopathies, thereby allowing treatment and/or prevention of the disease. The synucleinopathy to be treated and/or prevented may be, for example, Parkinson's Disease, Dementia with Lewy Bodies, Multiple System Atrophy or Alzheimer's Disease.

According to a fourth aspect of the invention there is provided a pharmaceutical composition comprising a peptide, peptide derivative or peptide analogue according to the first or second aspects of the invention with a pharmaceutically acceptable diluent.

The pharmaceutical composition may, for example, be formulated for systemic administration, such as by means of injection. Such a composition may for example be formulated as a sterile solution, for example a solution dissolved in sterile saline.

Alternatively the pharmaceutical composition may be formulated for oral administration, for instance as a liquid, capsules or tablets. In an alternate embodiment the pharmaceutical composition may be formulated for nasal administration, for example by means of a nasal spray or drops. The composition may also be formulated for transdermal application, for example by means of "patches".

Preferably between 1mg and 100mg of a peptide, derivative or analogue of the invention may be administered per day. Preferably the peptides, derivatives or analogues may be administered one to three times per week. Therapeutic use of peptides, derivatives or analogues of the invention may preferably be commenced prior to a patient developing a synucleinopathy. Therapeutic use may be maintained for as long as a patient is at risk of developing, or suffering from, a synucleinopathy.

According to a fifth aspect of the invention there is provided the use of a peptide, peptide derivative or peptide analogue according to the first or second aspects of the invention for the manufacture of a medicament for the prevention or treatment of a synucleinopathy.

According to the third, fourth and fifth aspects of the invention the synuclein the aggregation of which is to be inhibited or disrupted may be a wild-type form, or it may be a mutated, nitrated, phosphorylated or glycosylated form, or any other modified form.

The synuclein may be a synuclein fragment such as NAC. The synuclein may also be found as part of a complex with other molecules.

The peptides, peptide derivatives and peptide analogues of the invention may also be used to inhibit and/or disrupt the aggregation of  $\beta$ - or  $\gamma$ -synuclein.

The invention will be further described by way of example only with reference to the following non-limiting Examples, protocols and accompanying drawings which shows the results of the Examples 1 to 7.

## EXAMPLES

The following Examples were carried out using the protocols set out in Appendix 1.

### *EXAMPLE 1.*

The following Example was designed to investigate what part, or parts, of the  $\alpha$ -synuclein sequence were capable of binding to the full-length  $\alpha$ -synuclein molecule. Since amyloidogenesis is mediated by the binding of multiple  $\alpha$ -synuclein molecules to one another portions of the  $\alpha$ -synuclein sequence demonstrating a high capacity for binding to the full length molecule represent promising regions for use as contiguous sequences in accordance with the invention.

A large library of overlapping 7-mer peptides spanning the whole of the  $\alpha$ -synuclein sequence were created, such that the first peptide comprised residues 1 to 7 of  $\alpha$ -synuclein, the second peptide residues 2 to 8, the third peptide residues 3 to 9, etc. These peptides were tested for their ability to bind to the full-length  $\alpha$ -synuclein protein.

The results of the binding studies are shown in Figures 2 and 3 (in which the level of "background" binding has been subtracted from total binding). As these results illustrate, those peptides comprised of contiguous amino acid residues located within region 61-100 of  $\alpha$ -synuclein (that is to say peptides 61 to 95) were found to bind most tightly to the full-length protein. A further summary of the levels of binding of peptides, comprised of contiguous amino acid residues located within region 61-100 of  $\alpha$ -synuclein, to full length  $\alpha$ -synuclein is shown in Table 3.



**Table 3.**  
**Peptide Binding Results.**

<b>Peptide No</b>	<b>Peptide sequence</b>	<b>binding % to <math>\alpha</math>-synuclein</b>
Peptide 61	EQVTNVG	7.1
Peptide 62	QVTNVGG	5.1
<b>Peptide 64</b>	<u>TNVGGAV</u>	<b>12.8</b>
<b>Peptide 65</b>	<u>NVGGAVV</u>	<b>13.4</b>
Peptide 66	<u>VGGAVVT</u>	17.3
<b>Peptide 67</b>	<u>GGA VVTG</u>	<b>15.4</b>
Peptide 71	<u>VVTGVTA</u>	<b>16.2</b>
Peptide 73	<u>TGVTAVA</u>	14.7
<b>Peptide 74</b>	<u>GVTAVAQ</u>	<b>12</b>
Peptide 76	<u>TAVAOKT</u>	<b>15.7</b>
<b>Peptide 77</b>	<u>AVAQKT V</u>	<b>5</b>
Peptide 78	VAQKTVE	3.8
Peptide 79	AQKTVEG	4
Peptide 82	<u>TVEGAGS</u>	<b>18.2</b>
<b>Peptide 83</b>	<u>VEGAGSI</u>	<b>0</b>
Peptide 84	EGAGSIA	0
Peptide 85	GAGSIAA	0
Peptide 86	AGSIAAA	0
Peptide 87	GSIAAAT	1
Peptide 90	AAATGFV	5.3
Peptide 91	AATGFVK	10
Peptide 94	GFVKKDQ	7.9

**EXAMPLE 2.****Inhibition of NAC Aggregation**

The peptides numbers 1 to 5, listed in Table 2 (*supra*), were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides were tested for their ability to inhibit aggregation of NAC.

The peptides were dissolved in water and then diluted in 2x PBS. The final concentration of NAC was 100  $\mu$ M, either alone or with peptides according to the invention at ratios (NAC : peptide) of 2:1; 1:1 and 1:2. The samples were thereafter incubated at 37°C. Fibril formation was monitored by fluorescence of the dye Thioflavin T (ThT) (Sigma). Ten  $\mu$ l of the peptide solution was diluted into 190  $\mu$ l of PBS containing 20  $\mu$ M ThT. Fluorescence was measured in black plates, with excitation at 440 nm and emission at 490 nm using a Victor 1420 multi-label microtitre plate reader (Wallac).

The results of these experiments are shown in Figures 4 to 9.

**EXAMPLE 3.****Inhibition of  $\alpha$ -synuclein aggregation.**

Peptides and peptide derivatives listed in Table 2 (*supra*) were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides and derivatives were tested for their ability to inhibit the aggregation of  $\alpha$ -synuclein, as assessed by Th-T assay.

The peptides, or derivatives, were dissolved in water and then diluted in 2x PBS. The final concentration of  $\alpha$ -synuclein was 50  $\mu$ M, either alone or with peptides or derivatives according to the invention at ratios ( $\alpha$ -synuclein : peptide) of 2:1; 1:1 and 1:2. The samples were thereafter incubated at 37°C for four days. Ten  $\mu$ l of the peptide, or derivative, solution was diluted into 190  $\mu$ l of PBS containing 20  $\mu$ M ThT.

Fibril formation was monitored by fluorescence of the dye Thioflavin T (ThT) (Sigma). Fluorescence was measured in black plates, with excitation at 440 nm and emission at 490 nm using a Victor 1420 multi-label microtitre plate reader (Wallac).

The results of these experiments are shown in Figure 10.

It can be seen that of the peptides and derivative (inhibitor number 7) tested, all but inhibitor 4 caused significant inhibition of  $\alpha$ -synuclein aggregation.

**EXAMPLE 4.****Inhibition of  $\alpha$ -synuclein oligomerisation.**

Peptides and peptide derivatives listed in Table 2 (*supra*) were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides and derivatives were tested for their ability to inhibit the oligomerisation of  $\alpha$ -synuclein, as assessed by ELISA.

The peptides, or derivatives, were dissolved in water and then diluted in 2x PBS. The final concentration of  $\alpha$ -synuclein was 50 $\mu$ M, either alone or with peptides or derivatives according to the invention at ratios ( $\alpha$ -synuclein : peptide) of 2:1; 1:1 and 1:2. The samples were thereafter incubated at 37°C for four days. The peptide, or derivative, solution was diluted 1/100 in PBS and tested by the ELISA.  $\alpha$ -synuclein oligomerisation was monitored by ELISA as set out in Appendix 1. The results of these experiments are shown in Figures 11.

It can be seen that of the peptides and derivative (inhibitor number 7) tested, all but inhibitor 4 caused significant inhibition of  $\alpha$ -synuclein oligomerisation.

**EXAMPLE 5.****Inhibition of  $\alpha$ -synuclein aggregation (Western blotting analysis).**

Peptides 1 and 3 listed in Table 2 (*supra*) were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides and derivatives were tested for their ability to inhibit the aggregation of  $\alpha$ -synuclein as assessed by Western blotting.

Fresh or aged samples of  $\alpha$ -synuclein solution (50  $\mu$ M) were prepared. Further samples of fresh  $\alpha$ -synuclein solution were incubated for four days in PBS with peptide inhibitors 1 and 3 at a concentration of 100 $\mu$ M inhibitor : 50 $\mu$ M  $\alpha$ -synuclein. All samples were subjected to Western blotting analysis in accordance with the protocol set out in Appendix 1.

The results of these experiments are shown in Figure 12.

The protein band situated at 15kDa represents the  $\alpha$ -synuclein monomer (non-aggregated). The higher molecular weight bands are the result of  $\alpha$ -synuclein aggregation. The majority of  $\alpha$ -synuclein contained in the fresh sample is present in the form of the monomer. In contrast, the majority of  $\alpha$ -synuclein contained in the aged sample is present in aggregated form. That the  $\alpha$ -synuclein present in lanes 3 and 4 is present in predominantly monomeric form illustrates that both of the peptides tested caused significant inhibition of  $\alpha$ -synuclein aggregation.

**EXAMPLE 6.****Inhibition of  $\alpha$ -synuclein fibril formation.**

Peptides 1, 2 and 3 listed in Table 2 (*supra*) were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides were tested for their ability to inhibit  $\alpha$ -synuclein fibril formation, as assessed by electron microscopy analysis.

Electron microscopy was conducted in accordance with the protocol set out in Appendix 1. Fibril formation was investigated in aged 50 $\mu$ M  $\alpha$ -synuclein solution, and in samples of fresh  $\alpha$ -synuclein solution incubated for four days in PBS with the selected peptide inhibitors at a concentration of 100 $\mu$ M inhibitor : 50 $\mu$ M  $\alpha$ -synuclein. The results are shown in Figure 13.

Panel A of the Figure illustrates fibril formation in the aged  $\alpha$ -synuclein solution without inhibitors. Fibrils can be seen to have aggregated, creating large  $\alpha$ -synuclein accumulations.

Panels B, C and D illustrate fibril formation in the presence of inhibitors 1, 2 and 3 respectively. It can be seen that fibril formation is much reduced in the presence of all inhibitors tested, and that fibril formation has not occurred in the presence of inhibitors 1 and 2.

**EXAMPLE 7.****Cytotoxicity of peptides or derivatives.**

Peptides 1, 2, 3, 4 and 8 listed in Table 2 (*supra*) were synthesised using the peptide synthesis protocol described in Appendix 1. The peptides were tested for cytotoxic effects using the MTT assay, the protocol for which is described in Appendix 1.

The results of the experiments are shown in Figure 14. A reduction in the percentage of MTT reduction occurring indicates that the tested peptide has cytotoxic effects.

As can be seen from Figure 14, the inhibitors tested showed little cytotoxic activity at a range of concentrations between 0.01  $\mu$ M and 5  $\mu$ M.

## **APPENDIX 1.**

### **PROTOCOLS.**

#### **1. Design and Synthesis of Peptides**

##### **a. Biotinylated Peptide Library Synthesis**

All Fmoc protected amino acids were purchased from Advanced Chemtech Europe (Cambridge, UK), Fmoc- $\epsilon$ Ahx-OH was purchased from CN Biosciences UK (Nottingham, UK), and d-Biotin from Sigma-Aldrich Ltd. (Dublin, Ireland). The PAL Support amide resin and *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium Hexafluorophosphate *N*-oxide (HATU) were purchased from PE Applied Biosystems (Cheshire, UK); Piperidine, DIEA, DCM and DMF all from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

Multiple peptide synthesis was carried out at 0.010 mmol scale using an Advanced Chemtech 396 peptide synthesiser (Advanced Chemtech Europe, Cambridge, UK), using standard Fmoc chemistry. A 6-fold excess of amino acid, aminohexanoic acid and d-Biotin was used, and coupling was achieved using HATU. Cleavage of the peptides was carried out automatically by the machine over a 2 hours incubation at room temperature with a mixture of thioanisole, ethanedithiol, triisopropylsilane, water and TFA (2:1:1:1:95 v/v/v/v/v).

Peptide purity was ascertained by reverse phase HPLC using a C5 reversed phase 250 mm x 4.6 mm column (Phenomenex); peptide identity was verified using a Finnigan LCQ Ion Trap Mass Spectrometer.

The binding of biotinylated peptides produced according to the above protocol to full length  $\alpha$ -synuclein was investigated using protocol 2 below.



**b. Peptide synthesis**

All Fmoc protected amino acids, Fmoc PAL Support amide resin, Fmoc-L-Val-PEG-PS resin, HATU, DIEA, DMF, NMP, Piperidine and DCM, were purchased from PE Applied Biosystems (Cheshire, UK).

Peptides listed in Table 2 (*supra*) were synthesised at 0.1 mmole scale using an Applied Biosystem 433 Peptide Synthesiser using standard Fmoc chemistry. A 10-fold excess of amino acid was used, and coupling was achieved using HATU. Cleavage of the peptides was carried out over a 2 hour incubation at room temperature with a mixture of thioanisole, ethanedithiol, triisopropylsilane, water and TFA (2:1:1:1:95 v/v/v/v/v).

Peptide purity was ascertained by reverse phase HPLC using a C5 reversed phase 250 mm x 4.6 mm column (Phenomenex); peptide identity was verified using a Finnigan LCQ Ion Trap Mass Spectrometer.

**2. Binding Studies**

1. Recombinant  $\alpha$ -synuclein (100  $\mu$ l of 14  $\mu$ g/ml) in 200 mM NaHCO<sub>3</sub>, pH 9.6, was immobilized on Maxisorb microtiter plates (Nunc) over night at 4 °C.
2.  $\alpha$ -synuclein was aspirated from the plate by inverting over the sink. Plates were then washed 4 times with PBS-Tween 20 (0.05%) (PBST).
3. 100  $\mu$ l per well of blocking buffer (PBS buffer (pH 7.4) containing 2.5% gelatin (Sigma) and 0.05% Tween 20 (Sigma)) was added, and the plates were incubated for 2 hours at 37°C. Plates were then washed 4 times with PBST.
4. 100  $\mu$ l of biotinylated peptide was added per well (giving a concentration of 1000 pmole/well). The plate was covered and incubated for 2 hrs at 37°C.
5. Plates were then washed 4 times with PBST.
6. 100  $\mu$ l of ExtrAvidin-Alkaline phosphatase (Sigma) diluted 3:5000 in blocking buffer was then added per well, and incubated for 1 hr at 37°C.

7. Finally plates were washed 4 times in PBST. 100  $\mu$ l of Alkaline phosphatase Yellow "pNPP" was added per well and incubated for 30 min at RT. Absorbance values were then read at 405 nm.

## **2. Inhibition of $\alpha$ -Synuclein Aggregation**

Recombinant  $\alpha$ -synuclein protein was dissolved in PBS to a final concentration of 50  $\mu$ M, either alone or with peptides according to the invention at ratios ( $\alpha$ -synuclein : peptide) of 2:1; 1:1 and 1:2. The samples were thereafter incubated at 37°C for the required time in an Eppendorf Thermomixer with continuous shaking (1000 rpm). Fibril formation was monitored by fluorescence of the dye Thioflavin T (ThT) (Sigma). Ten  $\mu$ l of the peptide/ $\alpha$ -synuclein solution was diluted into 190  $\mu$ l of PBS containing 20  $\mu$ M ThT. Fluorescence was measured in black plates, with excitation at 440 nm and emission at 490 nm.

## **3. Inhibition of $\alpha$ -Synuclein Oligomerisation**

The following protocol may be used to investigate the ability of peptides, peptide derivatives or peptide analogues to inhibit the formation of soluble oligomers of  $\alpha$ -synuclein. These soluble oligomers form during the early stages of synuclein aggregation.

### **A. REAGENTS**

#### **i. Coating buffer**

200 mM NaHCO<sub>3</sub> (Sigma) pH 9.6. The solution is then filtered through a 0.2  $\mu$ m filter (Aldrich).

#### **ii. Blocking solution**

The blocking solution consists of 100 ml of PBS buffer (pH 7.4) containing 2.5% gelatin (Sigma) and 0.05% Tween 20 (Sigma). This solution should be made fresh and used in the same day and does not require filtering.

**iii. Biotinylation of antibodies**

Antibodies were biotinylated as recommended by the manufacturer "PIERCE".

**iv.  $\alpha$ -Synuclein solution**

The samples of  $\alpha$ -synuclein solutions with or without peptides of the invention were prepared as described in Section 2 above, to produce a final concentration of 50 $\mu$ M  $\alpha$ -synuclein.

**B. METHOD**

- i). Coat plate (Nunc, Maxisorp) with 100  $\mu$ l/well of the  $\alpha$ -synuclein (211) (mouse monoclonal IgG antibody which recognises amino acid 121-125 of  $\alpha$ -synuclein (Santa Cruz Biotechnology, Inc. Cat. No sc-12767) 1:200 (1  $\mu$ g/ml) in 200 mM NaHCO<sub>3</sub> (pH 9.6). The plate is then covered with a plate sealer (Sigma) and cling-film and stored overnight at 4°C.
- ii). Aspirate  $\alpha$ -synuclein (211) antibody from the plate by inverting over the sink. Wash 4 times with PBS-Tween 20 (0.05%) (PBST).
- iii). Block the plate by using 200  $\mu$ l /well blocking buffer. Incubate the plate at 37°C for 1 hr.
- iv). Wash the plates 4 times with PBST.
- v). Add 100  $\mu$ l/well of diluted  $\alpha$ -synuclein solution (final concentration 0.5 $\mu$ M in PBS). Cover the plate and incubate for 2 hrs at 37°C.
- vi). Wash the plate 4 times with PBST. Dilute the biotinylated  $\alpha$ -synuclein (211) 1:200 (1  $\mu$ g/ml) in blocking buffer and add 100 $\mu$ l /well. Incubate for 2 hrs at 37°C.
- vii). Wash 4 times with PBST.

viii). Add 100 µl/well of ExtrAvidin-Alkaline phosphatase (Sigma) diluted 3:5000 in blocking buffer and incubate for 1 hr at 37°C.

ix). Wash the plate 4 times in PBST. Add 100 µl /well of Alkaline phosphatase Yellow "pNPP" and incubate for 30 min at RT. Read absorbance values at 405 nm.

#### **4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-Page) and immunoblotting**

Fresh and aged samples of  $\alpha$ -synuclein solutions with or without peptide inhibitors were diluted in NuPAGE sample buffer (Invitrogen Ltd. UK), and were separated on NuPAGE Bis-Tris 4-12%, 1mm gels (Invitrogen Ltd. UK). The separated proteins were transferred to nitrocellulose membranes (0.45 µm; Invitrogen Ltd. UK) using wet transfer techniques. Membranes were blocked with 5% dried skimmed milk, dissolved in PBS-Tween 20 (0.05%) (PBST), for 60 min. The membranes were probed with primary antibody 211 (which recognises  $\alpha$ -synuclein (121-125) (Santa Cruz Biotechnology)) for overnight at 4°C. The membranes were washed several times with PBST, followed by incubation with HRP-conjugated goat anti-mouse (Dako) for 60 min. The protein bands were visualised using ECL reagents (Pierce, USA) as described by the manufacturer.

#### **5. Electron Microscopy (EM)**

Electron micrographs were produced from aged  $\alpha$ -synuclein solutions with or without peptide inhibitors. Ten µl of the samples were fixed with glutaraldehyde, stained with uranyl acetate, and examined on a JEOL JEM-1010 transmission electron microscope.

## **6. Cell Viability Assay**

The toxicity of the peptide inhibitors 1, 2, 3, 4 and 8 on cells was assessed by measuring cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). BE(2)-M17 human neuroblastoma cells were plated at a density of 25,000 cells per well on 96-well plates in 100  $\mu$ l fresh medium [i.e. DMEM (Gibco) containing 10% FBS (Gibco), 1% L-Glu (Gibco), 1 PncI/Strp (Gibco), 1% G-418 (Roche)]. Next day the medium was exchanged with 100  $\mu$ l fresh medium (as control) or 100  $\mu$ l of medium containing peptide inhibitors at concentrations ranging from 0.01  $\mu$ M to 5  $\mu$ M, and the cells were incubated at 37°C overnight. 10  $\mu$ l of stock MTT (6mg/ml) in PBS (Gibco) was added to a final concentration of 0.5 mg/ml, and the incubation was continued for another 4.5 h. Cell lysis buffer (100  $\mu$ l/well: 20% SDS (Sigma), 50% *N,N*-dimethylformamide (Sigma), pH 4.7) was added and incubated overnight at 37°C. Absorbance values at 570nm were determined with a Wallace plate reader.

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## CLAIMS

1. A peptide comprising a sequence of three to twelve contiguous amino acid residues (the "contiguous sequence") from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein, linked at the N-terminal and/or C-terminal end of the sequence to one or more further amino acid residue(s) (the "replacement" amino acid) which are more hydrophilic than the amino acid residue (the "substituted" amino acid) to which that end of the sequence is linked in naturally occurring  $\alpha$ -synuclein.
2. A peptide according to claim 1, wherein the "replacement" amino acid has a hydropathy index value at least one, preferably at least two, more preferably at least three, and most preferably at least four less than that of the "substituted" amino acid.
3. A peptide according to claim 1 or claim 2, wherein the "replacement" amino acid is a hydrophilic amino acid, as defined by its hydropathy index.
4. A peptide according to any preceding claim, wherein the contiguous sequence comprises a maximum of seven amino acid residues, more preferably a maximum of six amino acid residues, even more preferably a maximum of five amino acid residues, and most preferably a maximum of four amino acid residues.
5. A peptide according to any preceding claim, wherein the contiguous sequence includes at least two contiguous hydrophobic amino acid residues, as defined by their hydropathy index from amino acid residues 61 to 100 of  $\alpha$ -synuclein.
6. A peptide according to claim 5, wherein the contiguous sequence comprises at least three contiguous hydrophobic amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein.
7. A peptide according to claim 6, wherein the contiguous sequence comprises the amino acid residues alanine-valine-valine found at residues 69 to 71 of naturally

occurring  $\alpha$ -synuclein.

8. A peptide according to claim 6, wherein the contiguous sequence comprises the amino acid residues alanine-valine-alanine found at residues 76 to 78 of naturally occurring  $\alpha$ -synuclein.

9. A peptide according to claim 6, wherein the contiguous sequence comprises the amino acid residues isoleucine-alanine-alanine-alanine found at residues 88 to 91 of naturally occurring  $\alpha$ -synuclein.

10. A peptide according to any one of claims 1 to 4, wherein the contiguous sequence comprises at least four hydrophobic amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein.

11. A peptide according to claim 6, wherein the contiguous sequence comprises at least four contiguous hydrophobic amino acid residues from amino acid residues 61 to 100 of naturally occurring  $\alpha$ -synuclein.

12. A peptide according to claim 10, wherein the contiguous sequence comprises the amino acid residues alanine-valine-valine-threonine found at residues 69 to 72 of naturally occurring  $\alpha$ -synuclein.

13. A peptide according to any preceding claim, wherein the contiguous sequence is linked to one or more replacement amino acid(s) at one end of the sequence, the or each replacement amino acid being more hydrophilic than the corresponding amino acid in the naturally occurring  $\alpha$ -synuclein sequence.

14. A peptide according to any one of claims 1 to 12, wherein the contiguous sequence is linked to one or more replacement amino acid(s) at each end of the sequence, the replacement amino acids being more hydrophilic than the corresponding amino acids in the naturally occurring  $\alpha$ -synuclein sequence.

15. A peptide according to any preceding claim, comprising multiple contiguous sequences linked to one another by one or more replacement amino acid(s).
16. A peptide according to any preceding claim, further comprising an amino acid residue selected from the group comprising lysine and arginine at the N-terminal and/or C-terminal of the peptide.
17. A derivative or analogue of a peptide according to any one of claims 1 to 16.
18. A derivative or analogue according to claim 17, wherein the derivative is a D-amino acid derivative.
19. A derivative or analogue according to claim 17, wherein the derivative is an N-methylated amino acid derivative.
20. A derivative or analogue according to claim 17, wherein the analogue is a peptoid analogue.
21. A derivative or analogue according to any one of claims 17 to 20, wherein the derivative or analogue is more protease-resistant than the peptide from which it is derived.
22. A derivative or analogue according to any one of claims 17 to 20, wherein the derivative or analogue is adapted to facilitate its entry into biological cells, or across biological barriers (such as the blood brain barrier).
23. A derivative or analogue according claim 22, wherein the derivative is adapted by the addition of a membrane-permeable carrier peptide selected from the group comprising HIV-1 Tat-(48-60), flock house virus (FHV) coat-(35-49), *Drosophila* Antennapedia-(43-58), and basic peptides such as octa and hexa arginine peptides.

24. A method of treatment and/or prevention of a synucleinopathy comprising administering to a person in need of such treatment and/or prevention a therapeutically effective amount of a peptide, peptide derivative or peptide analogue according to any one of claims 1 to 23.

25. A method of treatment and/or prevention of a synucleinopathy according to claim 24, wherein the synucleinopathy to be treated and/or prevented is selected from the group comprising Parkinson's Disease, Dementia with Lewy Bodies, Multiple System Atrophy, or Alzheimer's Disease.

26. A pharmaceutical composition comprising a peptide, peptide derivative or peptide analogue according to any one of claims 1 to 23 with a pharmaceutically acceptable carrier.

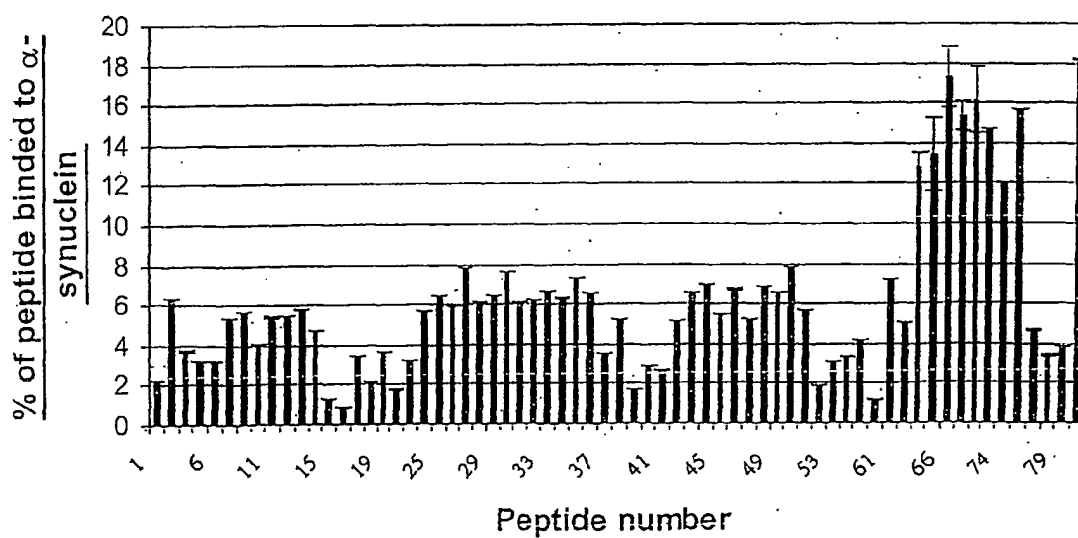
27. A pharmaceutical composition according to claim 26 wherein, the pharmaceutical composition is formulated for systemic administration.

28. The use of a peptide, peptide derivative or peptide analogue according to any one of claims 1 to 23 for the manufacture of a medicament for the prevention and/or treatment of a synucleinopathy.

MDVFMKGLSK	AKEGVVAAAE	KTKQGVAAEA	GKTKEGVLYV
GSKTKEGVVH	GVATVAEKT	EQVTNVGGAV	VTGVTAVAQK
TVGAGSIAA	ATGFVKKDQL	GKNEEGAPQE	GILEDMPVDP
DNEAYEMPSE	EGYQDYEP	(Sequence ID No. 1)	
EQVTNVGGAV	VTGVTAVAQK	TVGAGSIAA	ATGFV
(Sequence ID No. 2)			

## FIG. 1

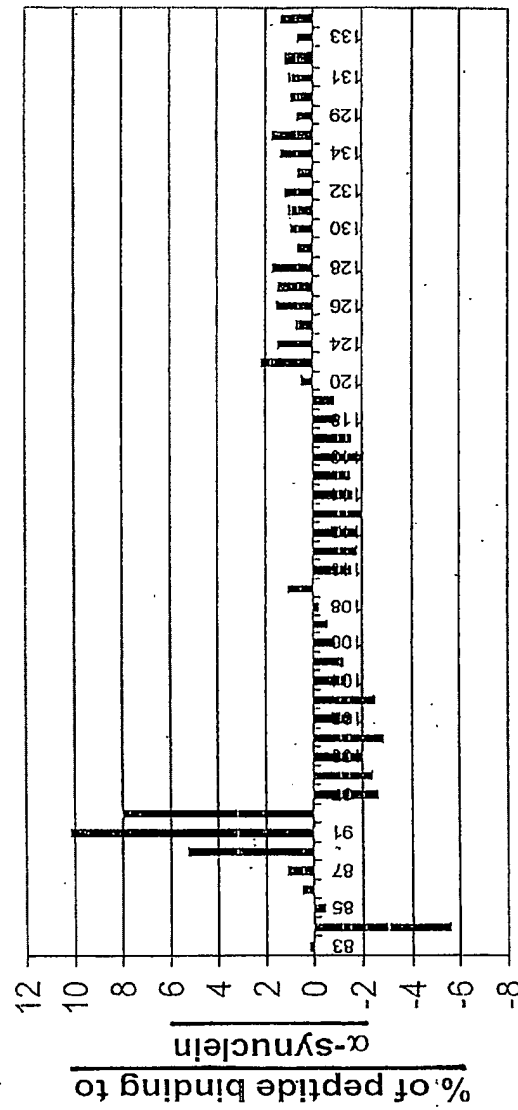
Binding of peptide library (1-82) to  $\alpha$ -synuclein  
protein



## FIG. 2

Binding of peptide library (83-134) to  $\alpha$ -synuclein

protein



peptide number

FIG. 3

Measuring the effect of Inhibitor1 (RGGAWTGR)  
on NAC (50  $\mu$ M) aggregation using Th-T assay

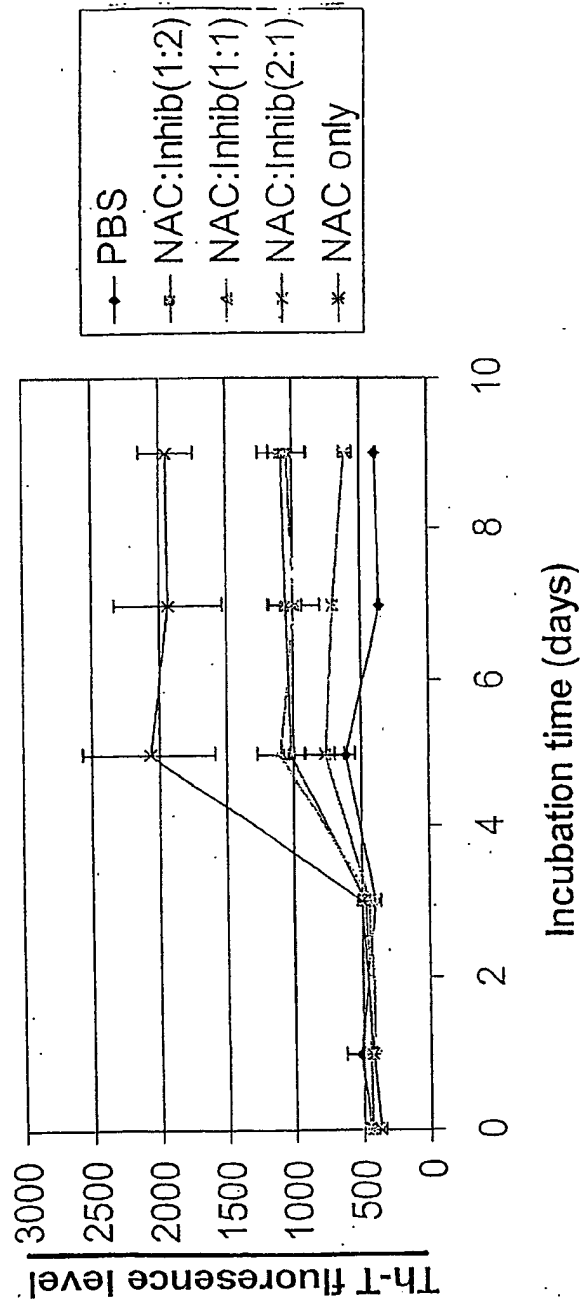


FIG.4



Measuring the effect of Inhibitor2 (RGAVVTGR) on  
NAC (50  $\mu$ M) aggregation using Th-T assay

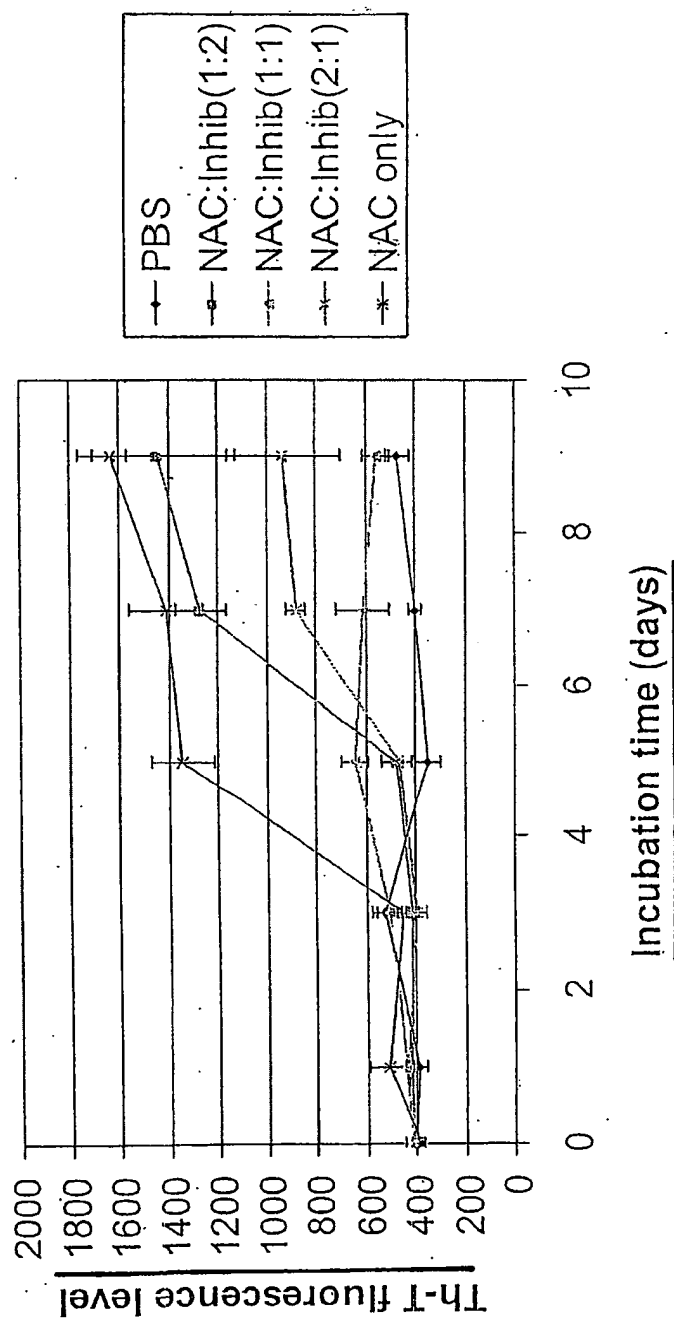


FIG.5

Effect of inhibitor3 (GGAVVTGR) on NAC (50  $\mu$ M)  
aggregation using Th-T assay

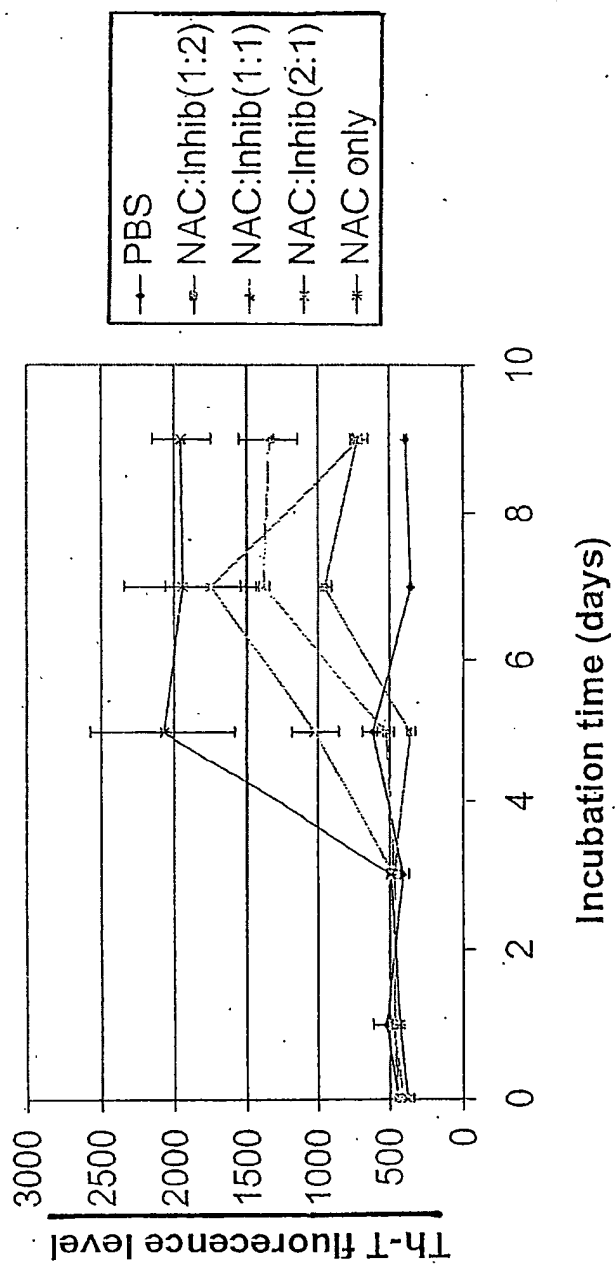


FIG. 6

Measuring the effect of inhibitor4 (RGAVVGR) on  
NAC (50  $\mu$ M) aggregation using Th-T assay

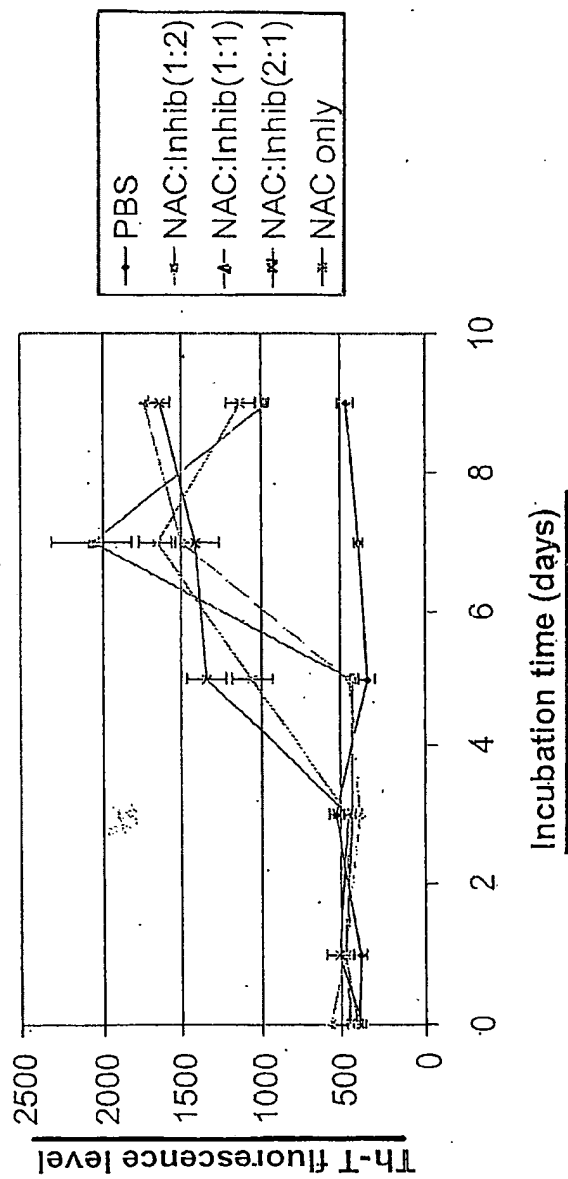


FIG. 7

Measuring the effect of inhibitor5  
(nGnGAVVTnGVTAVA) on NAC (50  $\mu$ M)  
aggregation using Th-T assay

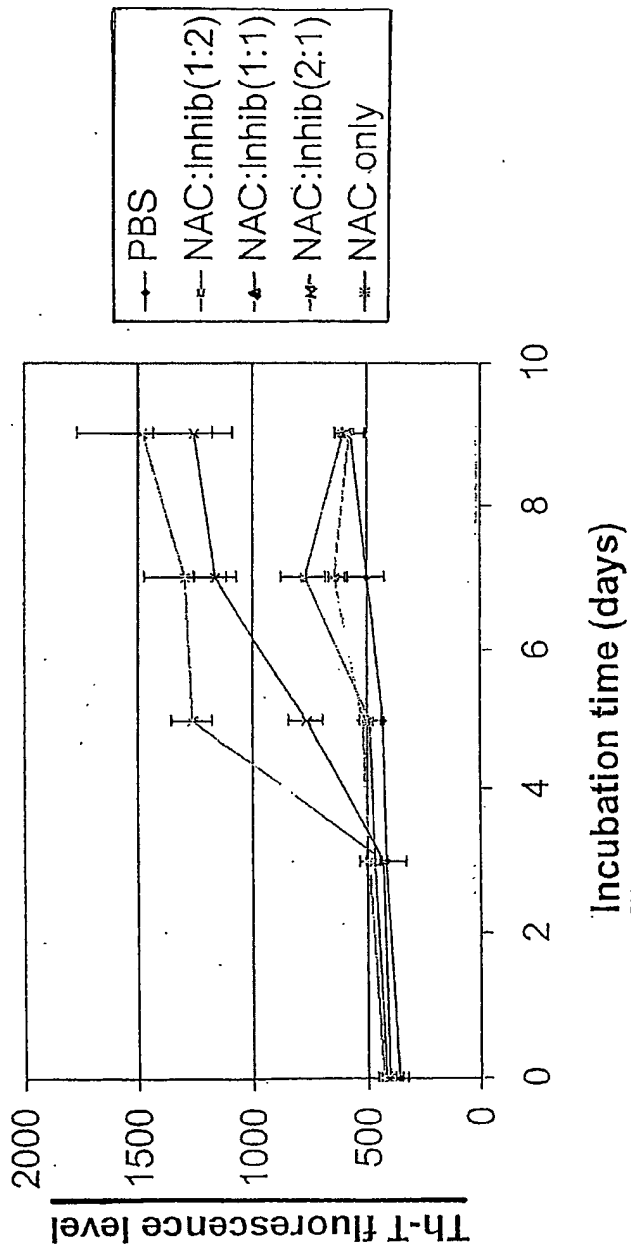


FIG. 8

Measuring the effect of inhibitor 6  
(nGGAVVTGVTAVA) on NAC (50  $\mu$ M)  
aggregation using Th-T assay

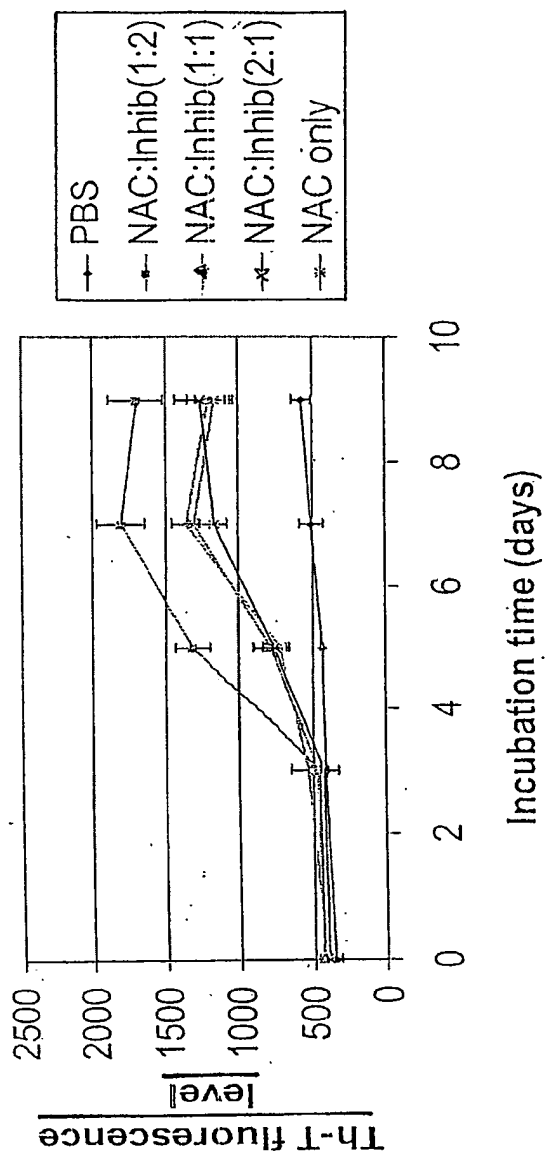


FIG. 9

Measuring the effect of peptide inhibitors on  
 $\alpha$ -synuclein (50  $\mu$ M) aggregation by Th-T assay

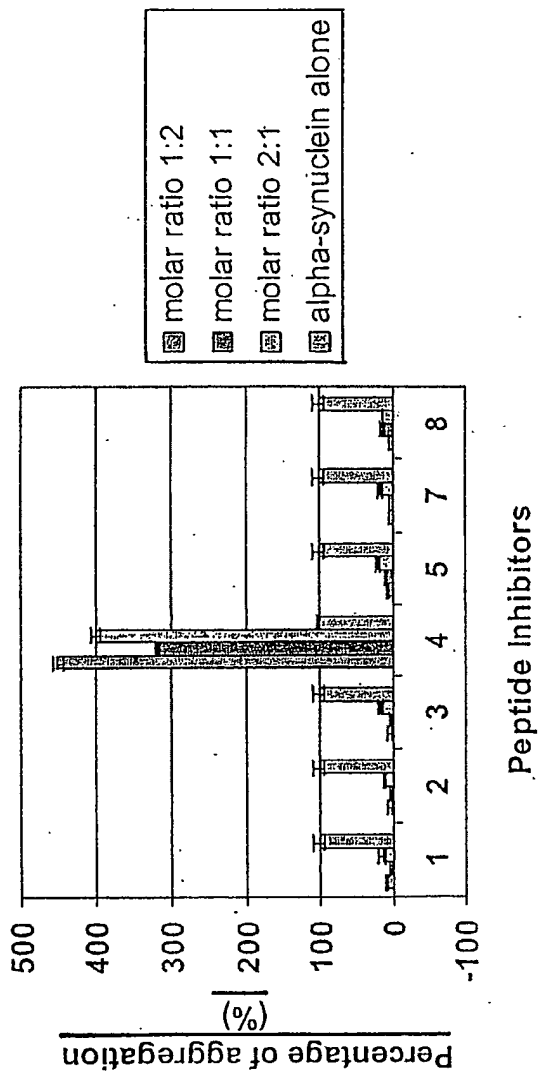


FIG.10

Fig. 11 Measuring the effect of peptide inhibitors on  $\alpha$ -synuclein (50  $\mu$ M) oligomerisation by ELISA assay

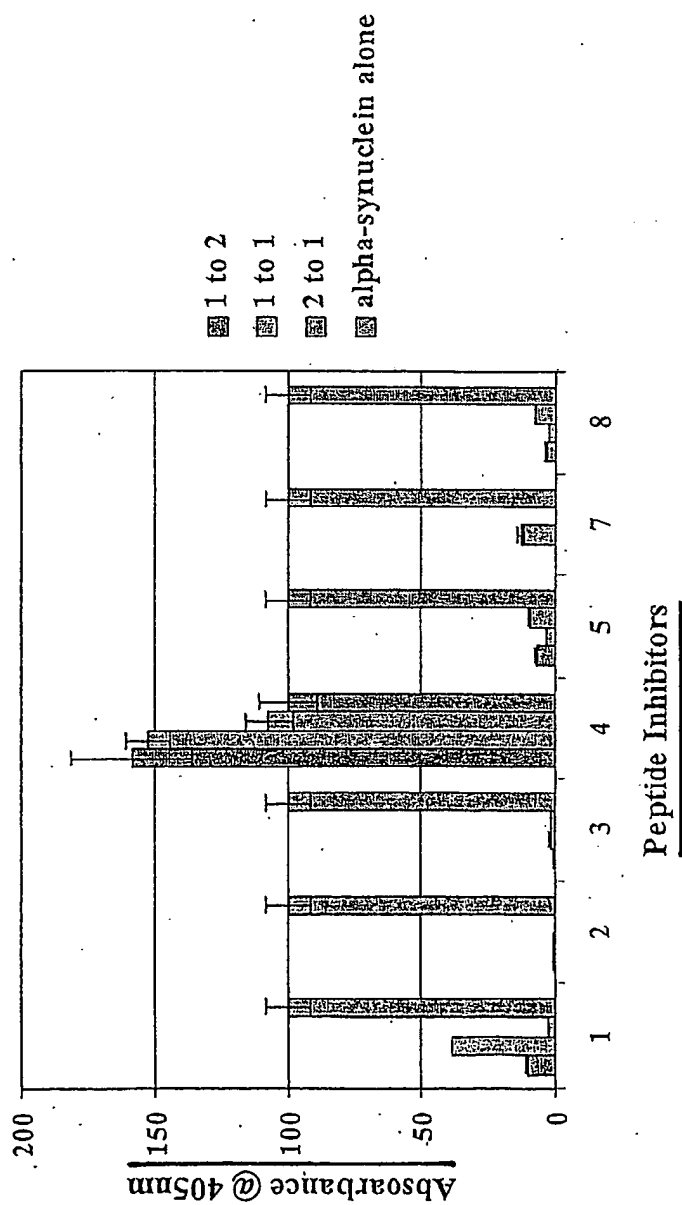
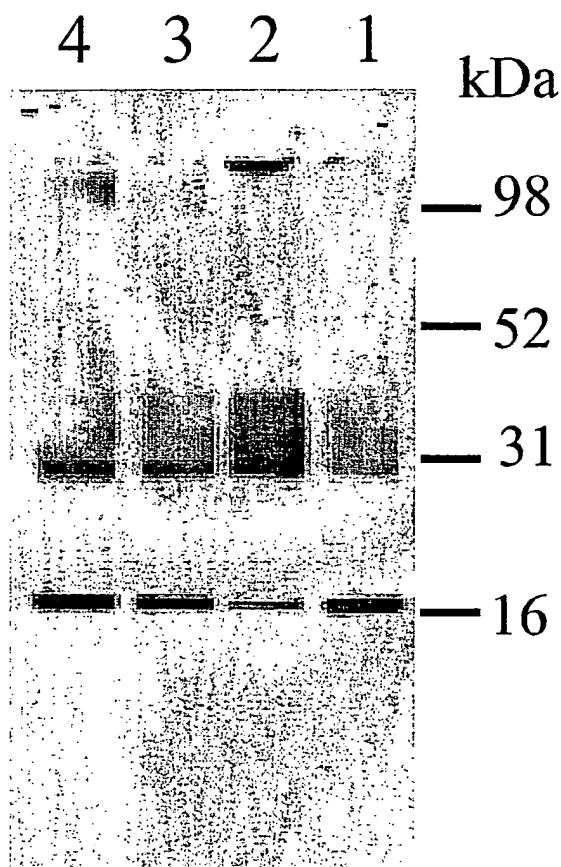


FIG.11

SDS-Page/Western Blotting: Analysis of the Effect of Inhibitors on  $\alpha$ -Synuclein Aggregation

Lane 1 Fresh  $\alpha$ -synuclein

Lane 2  $\alpha$ -Synuclein alone (50  $\mu$ M in PBS) was incubated for 4 days with continues mixing (1000 rpm) at 37°C.

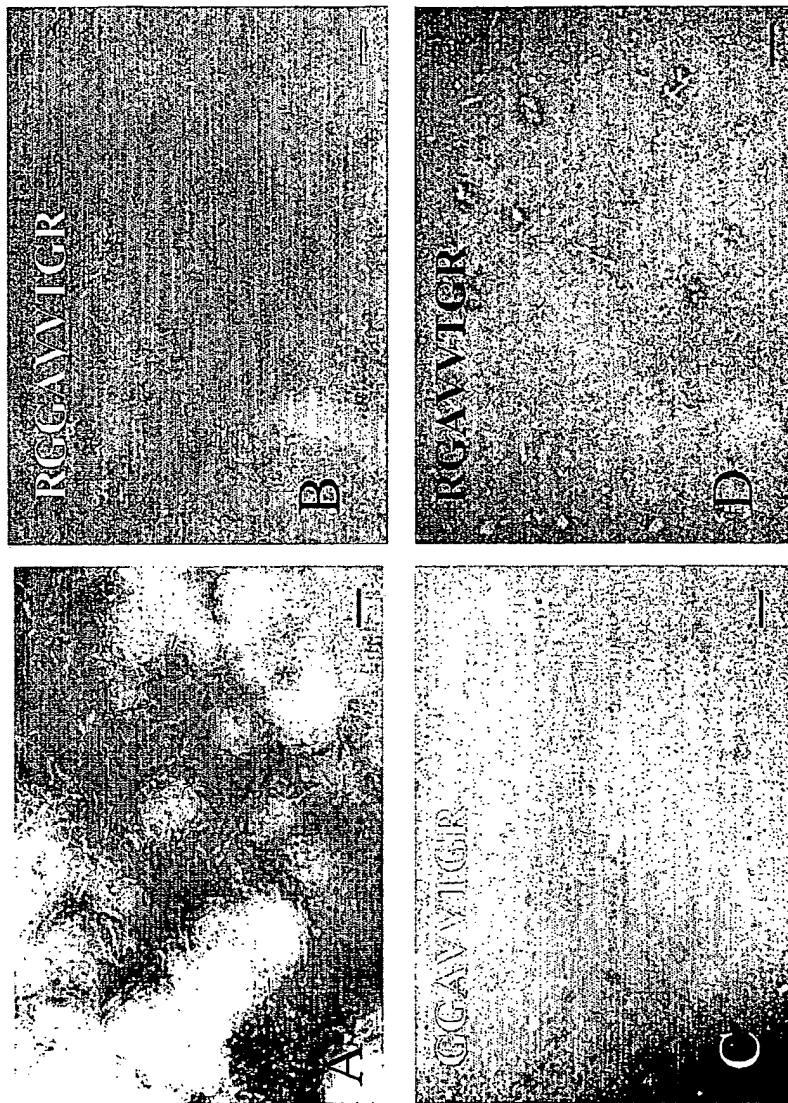
Lane 3  $\alpha$ -Synuclein with inhibitor1 at Molar ratio 1:2 (synuclein : inhibitor), were incubated for 4 days, with continues mixing (1000 rpm) at 37°C.

Lane 4  $\alpha$ -Synuclein with inhibitor3 at Molar ratio 1:2 (synuclein : inhibitor), were incubated for 4 days, with continues mixing (1000 rpm) at 37°C.

FIG.12



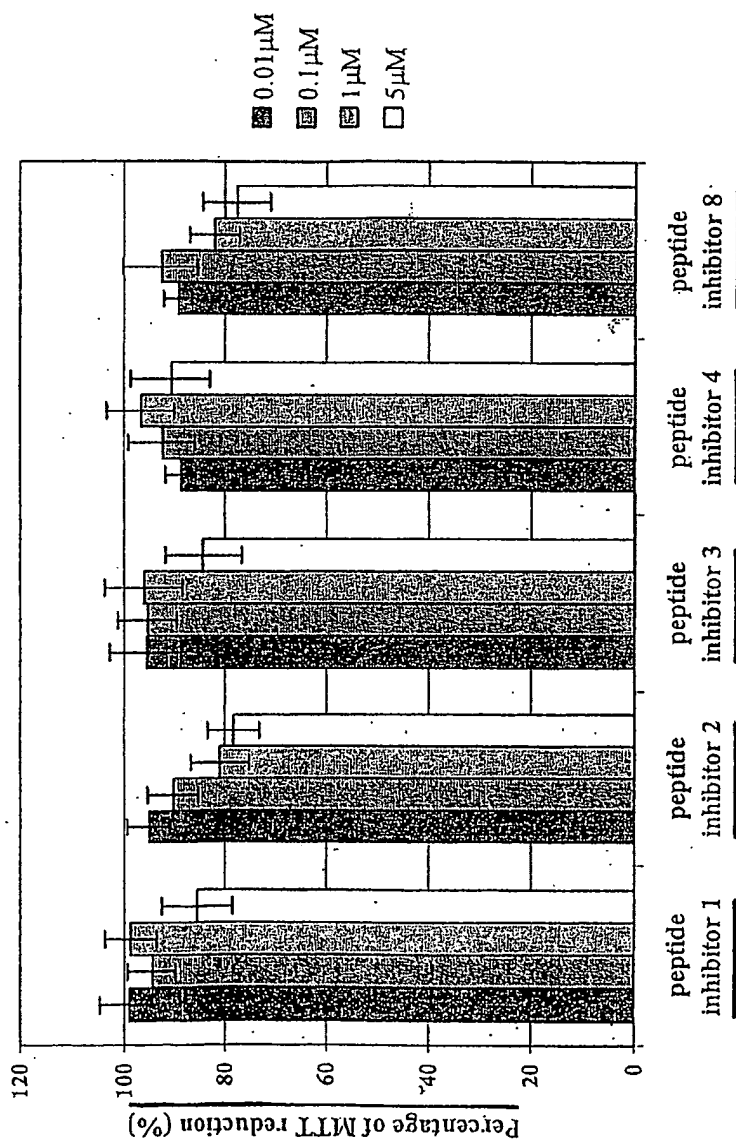
Electron Microscopy: Analysis of the Effect of inhibitors on  $\alpha$ -Synuclein Fibril Formation



$\alpha$ -Synuclein (50  $\mu$ M in PBS) alone (A) or with inhibitors (B, C, D) at Molar ratio 1:2 ( $\alpha$ -synuclein : peptide inhibitor), were incubated for 4 days, with continues mixing (1000 rpm) at 37°C.  
Scales bar = 100 nm

FIG.13

The effect of peptide inhibitors 1, 2, 3, 4 and 8 on  
BE(2)-M17 human neuroblastoma cells using MTT assay



**FIG. 14**